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Enhanced Tolerance to Low-K^+ Stress in Tobacco Plants, that Ectopically Express the CBL-interacting Protein Kinase *CIPK23* Gene

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Abstract

Xue G., Lu L.-M., Yang T.-Z., Li X.-H., Xing X.-X., Xu S.-X. (2016): Enhanced tolerance to low-K^+ stress in tobacco plants, that ectopically express the CBL-interacting protein kinase *CIPK23* gene. Czech J. Genet. Plant Breed., 52: 77–82.

Tobacco (*Nicotiana tabacum*) has a relatively high requirement for potassium (K^+). However, the molecular basis of tolerance to low-K^+ stresses in tobacco still remains unknown. Here, we report the role of a member of the *A. thaliana* CBL (calcineurin B-like) interacting protein kinase (CIPK) family, *AtCIPK23*, in low-K^+ stress responses in tobacco. Molecular analyses revealed that the *AtCIPK23* gene was successfully transferred into a tobacco cultivar K326 via *Agrobacterium tumefaciens*-mediated transformation. Overexpression of *AtCIPK23* in tobacco resulted in increased low-K^+ tolerance, which was demonstrated by higher dry biomass, longer primary root length, higher K^+ content and better growth status of transgenic tobacco plants compared to controls when both were treated in low-K^+ MS medium and low-K^+ hydroponics. Moreover, transgenic lines conferred tolerance to low-K^+ stress by increasing the K^+ uptake rate under low-K^+ conditions. Taken together, these results provide evidence that *AtCIPK23* may be involved in the CBL-CIPK signalling network in tobacco responses to low-K^+ stress.

Keywords: *AtCIPK23*; K^+ uptake; low-K^+ tolerance; potassium; tobacco

Potassium ions are the most abundant cations in plants and are important for various aspects of plant physiology, including cell expansion, enzyme homeostasis, salinity stress, and stomatal movements (Shabala & Pottosin 2014). The K^+ concentration in plant cell cytosol is maintained in the range of 100 mM (Leigh & Wyn Jones 1984). However, the concentration of free K^+ on the surfaces of plant roots in soils is usually below 1 mM (Shin 2014). Therefore, plants often suffer from the low-K^+ stress under natural conditions and display K^+-deficiency symptoms, typically leaf chlorosis and inhibition of growth and development (Ashley *et al.* 2006). Understanding the molecular mechanism underlying low-K^+ response and adaptation in plants will provide a platform for improving the crop tolerance to low-K^+ conditions (Cherel *et al.* 2014).

The uptake of K^+ and its redistribution throughout the plant are mediated by a number of potassium transporters (Wang & Wu 2013). *AKT1* (Arabidopsis K^+ Transporter1) has been identified as an inwardly rectifying K^+ channel in *Arabidopsis thaliana* and plays crucial roles in K^+ uptake from soil into root cells (Hirsch *et al.* 1998; Li *et al.* 2014). The *AKT1* channel has been shown to be post-translationally activated by a CBL interacting protein kinase (CIPK)-calcineurin B-like (CBL) protein complex at the plasma membrane (PM), where CIPK23 phosphorylates *AKT1* and activates AKT1-mediated K^+ uptake in *Arabidopsis* (Xu *et al.* 2006). Numerous investi-

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gations related to the CIPK family mainly have been studied extensively in various plants, including rice (*Oryza sativa*) (Yang *et al.* 2008), maize (*Zea mays*) (Tai *et al.* 2013), poplar (*Populus trichocarpa*) (Yu *et al.* 2007), cotton (*Gossypium hirsutum* L.) (He *et al.* 2013), apple (*Malus domestica*) (Wang *et al.* 2012), barley (*Hordeum brevisubulatum*) (Li *et al.* 2012), and so on.

Even though homologs that involve CBL-CIPK signalling have been identified from non-model plants, their roles in low-K^+ tolerance and molecular mechanisms are not very clear. Especially for tobacco, which is more sensitive to low K^+ availability than most of the other major field crops. So far only tobacco ectopically expressing the CIPK gene from *Arabidopsis*, wheat (*Triticum aestivum*) and chickpea (*Cicer arietinum*) has been characterized (Tripathi *et al.* 2009; Deng *et al.* 2013a, b). However, whether CIPK23 can confer low-K^+ tolerance in tobacco is still unknown.

Here, leaves of wild type (WT) tobacco (*Nicotiana tabacum* L. cv. K326) were transformed with *A. tumefaciens*-transformant strain GV3101 harbouring the binary plasmid pCAMBIA 1300 *35S::AtCIPK23* following the leaf disk co-cultivation protocol of Horsch *et al.* (1985). The visible shoots emerged in the co-cultivated explants (Figure 1A) after 10–12 days in a selection medium containing 50 mg/l hygromycin (Figure 1B). Those that were rooted in the selection medium were regarded as transgenic candidates compared to the controls which had no root initiation (Figure 1C). All of these transgenic lines were transplanted into pots containing compost (Figure 1D). The transgenic lines exhibited typical cultivar morphology in the greenhouse (Figure 1E). A total of 18 transgenic lines (T1) were identified by hygromycin-resistance and PCR analysis using primers specific to *AtCIPK23*. The presence of the transgene in hygromycin-resistant plants was determined by amplification of a 750 bp DNA fragment with HPT-specific primers in six putative transgenic lines (Figure 2A). The three homozygous PCR positive lines (A3, A5 and A6) showed distinct transgene expression at the transcript level of *AtCIPK23* by Northern Blot and Western Blot assays (Figure 2B and C).

To elucidate *AtCIPK23*-overexpressing line responses to low-K^+ tolerance assays, the plantlets were transferred to the MS plate medium and hydroponic culture systems. The results showed that there were

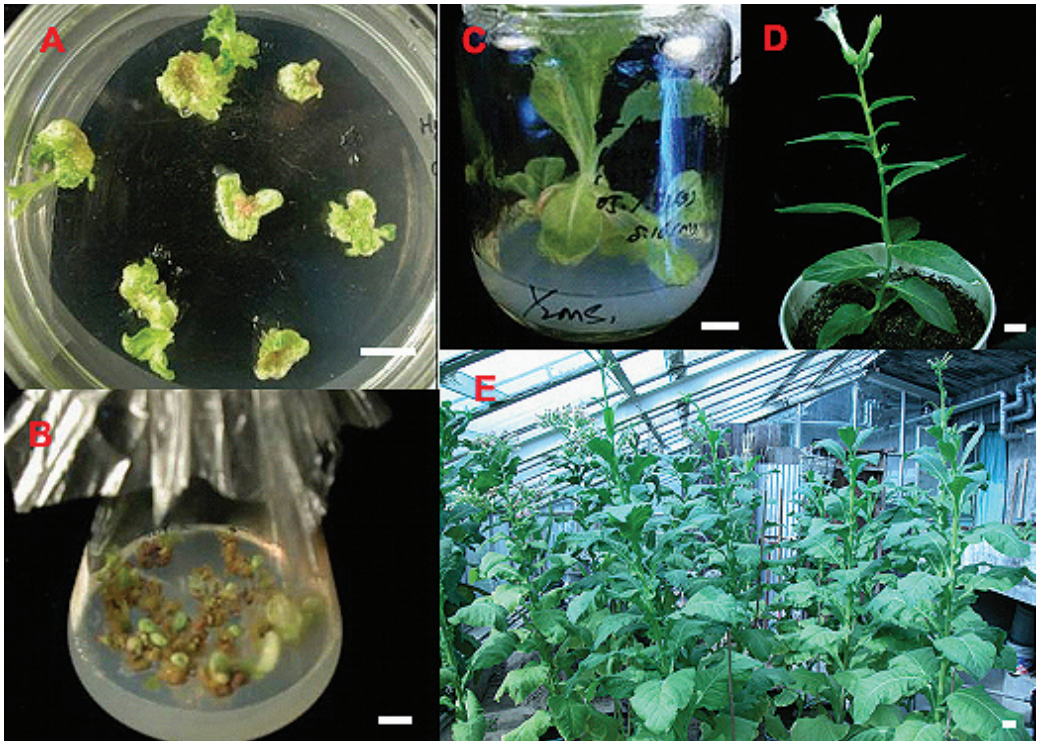

Figure 1. Tobacco transformation and identification of transgenic candidate lines: (A) recovered hygromycin-resistant callus after 6-week culture on the selection medium, (B) hygromycin-resistant callus dedifferentiated into embryos on the embryoid-induction medium, (C) recovered plantlets, (D) transgenic plantlets growing in the pot soil, (E) transgenic plant transferred to the greenhouse; scale bar – 10 mm

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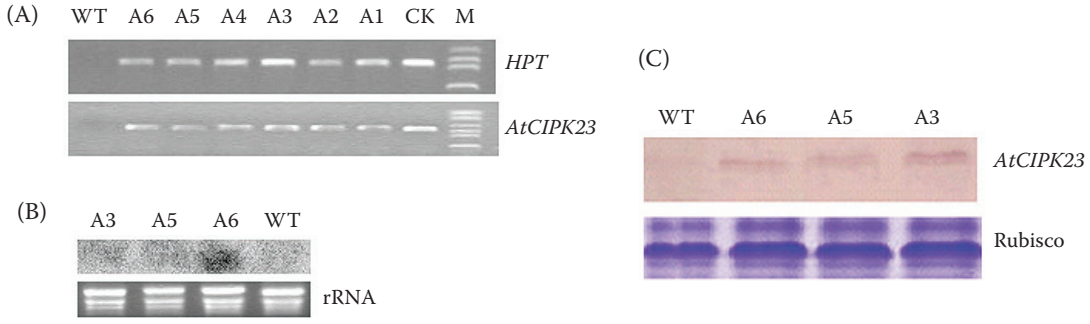

Figure 2. Molecular characterization of transgenic tobacco lines: (A) PCR analysis of the regenerated *HPT* and *AtCIPK23* gene positive plants, (B) Northern blot analysis of the *AtCIPK23* level, the lower panel in each section represents bands for ethidium bromide staining of RNA in a denaturing gel shown a quantitative loading control, (C) Western blotting analysis of the *AtCIPK23* level, the lower panel in each section represents bands for Rubisco (stained with Coomassie blue) to show equal loading of each sample; lanes: M – 100 bp DNA marker; WT – wild type; CK – positive plasmid control; A1–A6 – independent transgenic plants

no significant differences in phenotype between WT and its three transgenic lines when grown on the normal MS medium for 14 days or on Hoagland's nutrient solution for 30 days (Figure 3A and C). Nevertheless, A3, A5 and A6 showed absolute superiority to WT when grown either on low-K^+ MS medium for 14 days or in low-K^+ hydroponics for 30 days (Figure 3B and D). When grown on the low-K^+ MS medium, the transgenic lines (A3, A5, A6) exhibited significantly higher values of primary root length compared with non-transgenic lines (Figure 4). These growth characteristics are indicative of the low-K^+ tolerant phenotypes of transgenic lines.

We further investigated the plantlet dry weight and K^+ content in non-transgenic and transgenic lines (A3, A5 and A6) after growing for 30 days in two culture systems. The plant dry weight of the non-transgenic line was indistinguishable from that of the transgenic lines (A3, A5 and A6) in normal MS plate medium and hydroponic culture (Figure 3E). But in low-K^+ MS medium or in low-K^+ hydroponics, the plant dry weight of the non-transgenic line was significantly lower than that of every transgenic line (A3, A5 and A6) (Figure 3E). With regard to K^+ content, three transgenic lines had higher or significantly higher values than WT in LK, being in agreement with their less severe K deficiency symptom than in WT (Figure 3F). Expectedly, there were no differences in K^+ contents between transgenic lines and WT in MS (Figure 3F). These results of the K^+ content analysis indicate that *AtCIPK23* conferred to the transgenic lines increases of K^+ accumulation. It may be because *AtCIPK23* enhances the K^+ uptake ability of transgenic lines when subjected to low-K^+ stress. To test this possibility, we determined the K^+ uptake rates of the *AtCIPK23*-overexpressing lines and wild-type plants. As shown in Table 1, the *AtCIPK23*-overexpressing lines had the similar V_{max} (maximum velocity) compared to wild-type plants. The K_m (Michaelis-Menten constant) for K^+ uptake of three transgenic lines decreased to 63.43 mM, 79.25 mM and 113.59 mM, respectively, compared to 153.85 mM for wild-type plants. The results demonstrate that overexpression of *AtCIPK23* results in significant increases in the K^+ uptake affinity. Similarly, the C_{min} (minimum concentration) for K^+ uptake of the *AtCIPK23*-overexpressing lines was decreased compared to wild-type plants, which indicates that the transgenic plants may initiate K^+ uptake at much lower K^+.

To cope with environmental stimuli, plants have evolved precise regulatory mechanisms to perceive, transduce and respond to abiotic stresses that can negatively affect growth and development. Notably, increasing evidences have been provided for crucial functions of *CIPK23* in mediating hormone and stress

Table 1 Comparison of kinetic parameters of potassium uptake between various materials

Individuals	V_{max} (μmol/g FW/h)	K_m (μmol/l)	C_{min} (μmol/l)
WT	21.78	153.85	52.28
A6	18.19	63.43	15.58
A5	19.16	79.25	26.35
A3	21.94	113.59	36.70

V_{max} – maximum velocity; K_m – Michaelis-Menten constant; C_{min} – minimum concentration; WT – wild type; A3, A5 and A6 – three independent transgenic lines in the WT background

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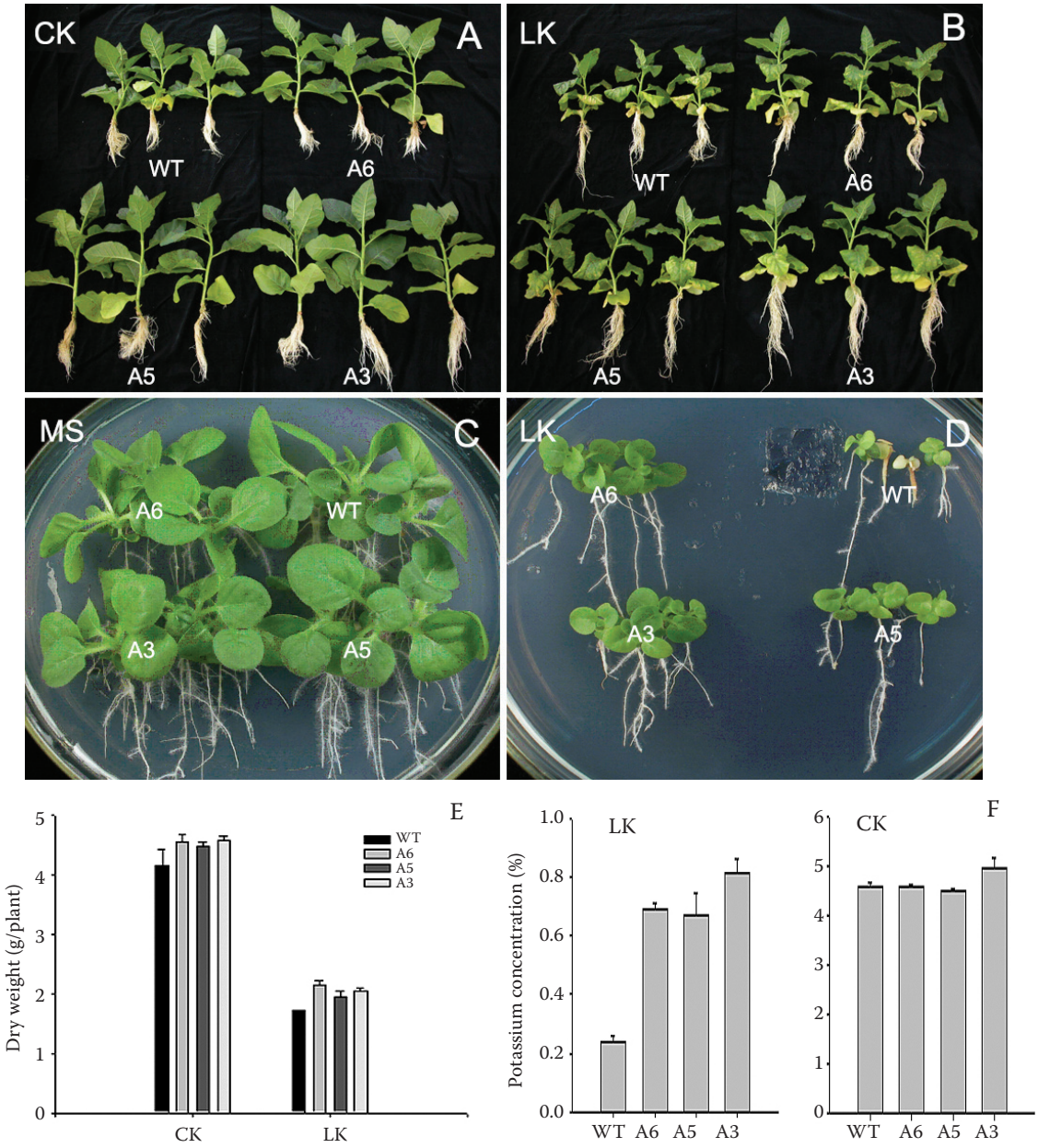

Figure 3. Phenotype assays of *AtCIPK23*-overexpression in tobacco: (A) and (B) phenotype comparison between wild type (WT) and its transgenic plants grown in normal hydroponics (CK, left) and LK (low K^+; 100 μM) hydroponics (right) for 30 days, (C) and (D) phenotype comparison between WT and its transgenic plants grown on normal MS medium (left) and on LK medium (right) for 14 days, (E) and (F) comparison of dry mass and K^+ content in normal hydroponics and LK hydroponics (right) for 30 days, respectively; A3, A5 and A6 – three independent transgenic lines in the WT background; plant phenotypes, dry weights and K^+ content – data are shown as means ± standard error (n = 4)

signalling responses in *Arabidopsis* (Cheong *et al.* 2007), rice (*Oryza sativa*) (Li *et al.* 2014), grapevine (*Vitis vinifera*) (Cuéllar *et al.* 2010) and poplar (*Populus trichocarpa*) (Zhang *et al.* 2010). In this study, we found that *AtCIPK23* overexpression remarkably enhanced tolerance to low-K^+ stress in transgenic tobacco. Similar results were obtained for *AtCIPK23* in potato (Wang *et al.* 2011). Enhancement of low-K^+ tolerance by *AtCIPK23* overexpression may be explained by increased K^+ uptake rates and K^+ affinity in the overexpressing plants. In transgenic plants, the primary root growth was not inhibited by a low-K^+ stress, in contrast to the controls. However, in normal K^+ conditions, the transgenic lines did not show any

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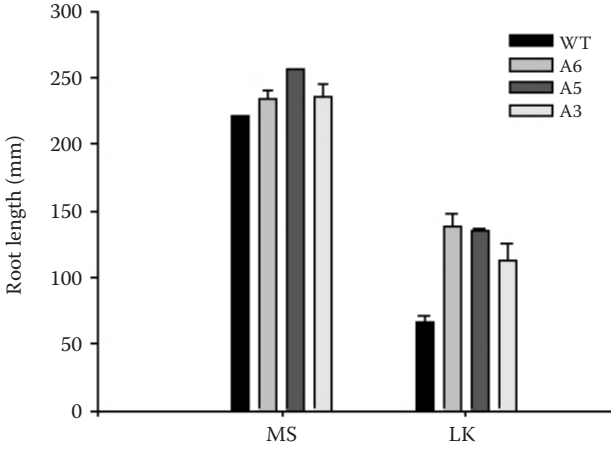

Figure 4. Comparison of the length of main roots of different material grown on MS and LK (low K^+; 100 μM) medium, respectively, for 8 days after having been grown on MS medium for 7 days; WT – wild type; A3, A5 and A6 – three independent transgenic lines in the WT background

advantages in K^+ accumulation compared with the non-transgenic line. This could be due to the fact that both non-transgenic and transgenic lines can absorb enough K^+ for normal and healthy growth, and that the absorption of K^+ in the roots is regulated by the demands of plants themselves (Shin 2014). Therefore, the contribution of the *AtCIPK23* gene to K^+ accumulation and K^+-use efficiency was decreased under the normal external K^+ level.

In conclusion, overexpression of *AtCIPK23* significantly enhances the tolerance to low-K^+ stress in transgenic tobacco, indicating that *AtCIPK23* acts as a positive regulator in response to low-K^+ stresses, and is supposed to be a potential candidate gene to improve stress tolerance by genetic manipulation in tobacco and other crops.

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